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Analyses of pre- and post-operative serum samples from 128 breast cancer patients for the 90kDa glycoprotein TAA-specific immune complexes (TAA-IC) and correlation of the results with clinical follow-up of these patients for five years revealed that the TAA-IC is useful in identifying breast cancer patients who harbor occult micrometastases, or are at high risk of developing recurrent disease after surgical resection of the disease. This assay, like most other tumor markers is not 100% accurate. There are some false postives and some false negatives; however, these false predictive values are less significant than many other markers commonly used. We have studied the immunobiology of the 90kDa glycoprotein TAA to the extent that the false postitive and negative results are reasonably explained. In addition, a new breast tumor-associated antigen (B-TAA) which is recognized by allogeneic serum samples from breast cancer patients has been identified in NP-40 extract of breast cancer cell lines. A lymphoblastoid cell line (LCL-4) from immortalization of a breast cancer patient's B-cells by EBV transformation has been established. This lymphoblastoid cell line continues to secrete IgM antibodies that react with breast cancer cell extracts and spent medium.

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Rishab K. Gupta 10/28/96

PI - Signature Date

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INTRODUCTION

Breast cancer is the number one cancer in women and its incidence has been increasing. The risk of developing cancer is increased further in women who have already had cancer in one breast or where there is a history of breast cancer in a mother or sister, and in women with benign breast disease. Oncologist are continually faced with the problem that by the time most breast cancers are clinically detectable, metastases have already occurred. As a result, many efforts have been made to develop techniques for early diagnosis and determination of prognosis. Once such techniques are developed, it will be possible to identify individuals who are at high risk of developing breast cancer or recurrent disease. With the identification of such individuals, it will be easier for the oncologist to place them in appropriate regimens for further diagnosis and treatment, and to design clinical trials for more realistic and significant evaluation of the therapeutic modalities. Also, since survival of breast cancer patients is short once the widespread disease is established, early diagnosis and treatment of these patients are important to increase survival. Therefore, there is a great need to develop sensitive and accurate assays that can aid the modern sophisticated breast cancer screening programs. With the availability of results from these assays, the oncologist will be in a better position to manage the disease before it metastasizes or becomes incurable. Accomplishment of this task should help achieve the objective of reducing breast cancer mortality among women and improve the quality of their life. Our approach is to develop immunologic assays to accomplish this task. We believe that development of sensitive immunologic methods, such as detection of breast TAA-specific IC that are in vivo immune reaction products between immunogenic breast TAA and corresponding antibodies.

It is now widely recognized that breast carcinomas, like other human neoplasms, express neoantigens (*1-6*). Undoubtedly, the detection of breast TAA potentially represents the most selective approach to immunoprognosis of the disease. However, in order for this approach to be feasible, first it must be established that the tumor in question does indeed have the specific breast TAA for which the assay is developed; and secondly, that these antigens are released into circulation in sufficient amounts to be detected by immunological assays. It should also be recognized that this approach may be limited by the inability of TAA to persist in the circulation.

There is adequate evidence to denote that certain antigens expressed by breast cancer cells are immunogenic in the host. As a result, breast cancer patients may have humoral antibodies. Upon appearance of the antigens due to subclinical tumor growth, humoral antibodies will react with these antigens and result in the formation of TAA-specific circulating IC. Thus, one would expect to find tumor antigen specific IC in the serum before free antigen could be detected. It has recently become clear that the majority of techniques available to quantify IC do not necessarily have direct value in the clinical screening or diagnosis of cancer patients because of their non-specific nature. Most of the reported observations consistently showed increased IC levels in the sera from cancer patients. With rare exceptions, many of these reports failed to identify the specificity of antigen or antibody constituents of the IC. The fact that increased levels of IC or like material that has been detected in cancer patients does not

necessarily mean that they were composed of tumor antigen and its corresponding antibody. For these reasons, it becomes necessary to determine the nature of the antigen(s) in the IC of IC-positive cancer patients or to develop antigen specific IC detection method(s) in order to accurately assess their clinical significance. Therefore, quantification of TAA-specific IC by sensitive methods should serve as a reliable marker for diagnosis and prognosis of breast cancer and for determination of tumoricidal effectiveness of various treatment modalities. Furthermore, detection of TAA-specific IC by the methods which we have developed gives an amplification effect and makes the assay more sensitive to determine the presence of TAA in a given sample.

We believe that the limited success achieved by various investigators in developing in vitro correlations of human tumor immunity that are prognostically useful was largely due to unavailability of well-characterized immune reagents. We realize that the problem of tumor-host interaction is extremely complex; however, we are convinced of the importance of tumor immunology in prognostication of the clinical course of breast cancer in humans. The key to our understanding more fully the problem is to work towards determining the clinical significance of the defined and refined human tumor antigens, and to determining the immune responses of the host to these antigens.

BODY

EXPERIMENTAL METHODS:

Serum and lymphocyte samples were obtained from breast cancer patients when they visited our clinic. The serum samples were kept frozen at -35C and lymphocytes were viably cryopreserved in liquid nitrogen in the specimen bank. The clinical information on these patients was maintained in a database. These retrospectively procured serum samples were used in the investigations described below to comply with the Statement of Work (SOW) of this grant.

In the previous grant period, we developed and optimized an ELISA to detect a 90kDa glycoprotein tumor-associated antigen (TAA) specific immune complexes in sera of breast cancer patients. This assay appeared to be as effective in detecting the autoimmunogenic 90kDa-TAA in sera of breast cancer patients as carcinoembryonic antigen (CEA) or CA15-3. Although, there were no positive or negative associations between either of these markers and 90kDa-TAA, at least one of the three markers was positive in 91% of breast cancer patients. The details of the assay were described in the last progress report.

Since we were able to reproducibily perform the assay and were able to obtain consistent results, we had proposed in the last progress report to continue our investigations to determine the clinical utility of the TAA-IC in diagnosis and prognosis by correlating positive results in clinically disease free breast cancer patients with subsequent recurrences on follow-up for at least 5 years.

PEG-IC assay to detect antigen non-specific immune complexes was performed as described by Riha et al (*7*) and Digeon et al (*8*). Comparision of the PEG-IC results with those of TAA-IC ELISA clearly denoted that the PEG-IC assay was not of any further significance in terms of increasing the incidence. Therefore, in light of the availablity of the TAA-IC we have discontinued the use of this assay for the time being.

In addition to TAA-specific-IC determination, the serum samples were also used as the source of antibody to determine the presence of antigen(s) in breast cancer and control cell line extracts by ELISA and Western blot technique.

Ninety serum samples from breast cancer patients taken postoperatively were used as the antibody source against ultrasonically disrupted breast cancer cell extract in a conventional ELISA. Wells of the ELISA plate were sensitized with 200 ug protein of the cell extracts. Ten serum samples from self-proclaimed healthy females were used as controls. Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent. The antibody titers of the sera from breast cancer patients ranged from less than 1:100 to 1:12,800; whereas, level of reactivity in the control normal sera was 1:300 or less. The serum samples were pre-adsorbed with human normal peripheral blood lymphocytes (equal packed cell volume) to eliminate any reactivity due to histocompatibility antigens.

Five breast cancer, one colon cancer, one melanoma, one sarcoma, and one normal fibroblastic cell lines were grown in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Cells were cultured as monolayer in T150 flasks. Cells were harvested by scrapping when growth reached about 80% confluency. The cells were washed five times with ice cold PBS (0.025M phosphate buffer supplemented with 0.15M sodium chloride at pH 7.2). The washed cells (5×10^6) were peletted and lysed with 1.0% NP-40 in 50mM Tris and 150mM sodium chloride buffer (pH 8.0) for 30 minutes. The lysed cells were centrifuged at 10,000 x g for 10 minutes at 4C. The supernate was further centrifuged at 36,000 x g for 30 minutes, and the clear supernatant was used as target antigen in Western blot (*9*) following separation by SDS-PAGE as described by Laemmli (*10*). Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent, and the reactivity was visualized by NBT/BCIP substrate. To obviate the problems due to non-specific protein-protein interactions, the target antigen was precleared with immobilized protein-A (Sigma Chemical Co., Saint Louis, MO).

One of the breast cancer patient's serum which exhibited high antibody level in ELISA was used to isolate and purify IgG antibodies by DEAE Affi-Gel blue column chromatography (*11*). Five ml serum was dialized against 0.02M K_2HPO_4 supplemented with 0.02% sodium azide at pH 8.0. The dialyzed serum was applied to a 20 ml bed volume of the gel equilibrated with the phosphate buffer. The column was flushed with 40 ml of the phosphate buffer. The effluent containing IgG was collected, concentrated to 5 ml by ultrafiltration and analyzed for IgG concentration by radial immunodiffusion. The IgG concentration was 6.1 mg/ml. This purified IgG antibody fraction was used for its reactivity to tumor (breast and other cancer) and normal cell lysates in Western blot.

Peripheral blood lymphocytes (PBL) were obtained from breast cancer patients at the time when they had high anti-breast antibody levels in ELISA. The lymphocytes were separated by Ficoll-Hypaque centrifugation and partially depleted of T lymphocytes and cytophilic Ig by incubation at 37C for 1 hr in RPMI by the AET-rosetting method (*12*). The enriched B-lymphocytes were suspended (10 x 10^6 cells/ml) in cell free culture filtrate of Epstein Baar virus (EBV) producing marmoset lymphoblastoid cells to transform with the EBV as described by Irie et al (*13*). After 24 hr incubation, the growth medium was replaced by RPMI-FCS medium. Supernates from wells with visual growth of hybrids was analyzed for human immunoglobulin secretion by an enzyme immunofiltration assay (*14*) and then tested for anti-breast TAA activity by ELISA.

Fisher's exact test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, Ca, was used to determine statistically significant differences among the post-operative 90kDa glycoprotein TAA-specific IC positive and negative breast cancer patients with respect to recurrent disease over a follow-up period of 60 months. All comparisions were two-tailed and a p value of less than 0.05 was considered statistically significant. Statistical analysis for disease free survival was performed by the Mantel-Cox method.

RESULTS:

APPLICATION OF 90kDa GLYCOPROTEIN TAA-IC ELISA TO ANALYZE PRE- AND POST-OPERATIVE SERUM SAMPLES:

From a group of 128 breast cancer that were positive for 90kDa TAA-IC pre-operatively, serum samples were obtained retrospectively 2 to 12 weeks after surgical resection of all of the accessible tumor. The serum samples (256 total) obtained before surgery and after surgery were analyzed by the TAA-IC ELISA. Of the 128 patients, 76 (59%) became negative after surgery for the marker, suggesting either a complete resection of the tumor or a reduction in tumor burden to a level below the threshhold level that could result in the serum to become TAA-IC positive. Despite surgical resection of the accessible tumor, there were 52 patients who remained positive for the 90kDa TAA-IC marker. Longitudinal clinical follow-up of the patients revealed that 34 of 52 (65%) post-operatively positive patients developed recurrent disease within five years. On the contrary, 9 of 76 (12%) of the post-operatively TAA-IC negative patients developed recurrent disease ($p < 0.05$). Thus, it is apparent that in the group of patients in whom the TAA-IC values remained positive, all of the disease was not resected and these patients harbored micrometastases. This translates to the fact that 90kDa glycoprotein TAA-IC marker can effectively identify patients with poor prognosis. Many patients who remained disease free for greater than 5 years were consistently negative for the TAA-IC (Figure 1) and patients who had developed recurrent disease either remained positive after removal of tumor or became positive for the marker before clinically detectable disease (Figure 2).

Statistical analysis of TAA-IC positive and negative patients by the Mantel-Cox method

revealed that the two groups differed significantly ($p< 0.05$) in terms of recurrence rate. The incidence of 90kDa TAA-IC positivity was not related to the duration of post-operative recurrence. The time to recurrence in TAA-IC positive patients ranged from 1 to 58 months with a mean $\pm$ SD of 29 $\pm$ 16 (Table I). These results again suggest a positive correlation between postoperative presence of TAA-specific IC and subclinical residual or recurrent disease.

CLINICAL UTILITY OF 90kDa GLYCOPROTEIN TAA-IC IN CONJUNCTION WITH RESULTS OF BIOPSY OF ABNORMAL MAMMOGRAPHY:

We are continuing to analyze serum samples taken at the time of biopsy of suspicious breast nodules that are identified by mammography. As depicted in Table II, the correlation of TAA-IC results with histopathological findings is excellent. Of the 151 abnormal mammograms, 75 patients had no malignancy by histopathology. These patients were considered clinically free of disease; however, 7% (5/75) sera of these patients was postive for TAA-IC. Eleven percent (17/151) patients had histopathologically proven ductal carcinoma in situ and 39% (59/151) had invasive ductal carcinoma. The incidences of TAA-IC in sera of these two groups of patients at the time of biopsy were 47% and 64%, respectively. Overall, 34% (51/151) patients with abnormal mammography were postive for the TAA-IC and 66% (100/151) were negative. These results clearly denote that the TAA-IC marker could be a valuable adjunct to mammographic screening of women.

REACTIVITY OF BREAST CANCER SERA WITH TUMOR CELL EXTRACT IN ELISA:

Figure 3 represents the reactivity of sera from a breast cancer patient and a normal individual against the extract of a breast cancer cell line in ELISA. Antibody titers in 100 serum samples from breast cancer patients ranged from <1:100 to >1:12,000; whereas, such titers in normal control sera were less than 1:300.

ISOLATION OF IgG BY DEAE AFFI-GEL BLUE:

As described in the methods section, an IgG fraction was prepared from a high titered serum from a breast cancer patient. The isolated fraction was devoid of other serum proteins that could be detected by immunoelectrophoresis using goat antibodies to whole human serum. The protein concentration of the isolated fraction was 6.1 mg/ml. This fraction was used as the antibody source in Western blot.

SDS-PAGE AND WESTERN BLOT ANALYSIS OF NP-40 EXTRACTS OF TUMOR AND FIBROBLASTIC CELL LINES:

Figure 4 depicts the Coomassie blue stainable protein heterogeneity of extracts of NP-40 lysed cells of breast cancer, colon cancer, sarcoma, melanoma, and normal fibroblasts. The extracts were prepared from 5 x 10^6 cells, and 20ul amounts of the extracts after reduction with 2- mercaptoethanol were loaded into lane of 6 to 20% gradient gels (Novex, San Diego, CA).

After electrophoresis, the gel was stained with Coomassie blue. Similar gels were run in duplicate; but after electophoretic separation, the proteins were electo-transferred to nitrocellulose. One of the nitrocellulose membrane after blocking with non-fat milk was reacted with 1:200 dilution (3ug IgG protein) of antibody from serum of a breast cancer patient (Figure 5), and the other was reacted with IgG fraction of the normal serum (Figure 6). While no appreciable reactivity was seen on the blot reacted with IgG from normal serum (Figure 6), a prominent band at about 53kDa region was visible in lanes loaded with breast cancer cell extracts but not in lanes loaded with other tumor cell or fibroblastic cell extracts. In the preliminary studies, this appears to be a breast tumor-associated antigen (B-TAA). Our subsequent efforts will be directed to isolate, purify, and physicochemically characterize this antigen with respect to epitope analyses.

ISOLATION OF B-CELLS FROM BLOOD OF BREAST CANCER PATIENTS AND TRANSFORMATION WITH EPSTEIN BARR VIRUS:

Peripheral blood lymphocytes (PBL) were isolated from ten breast cancer patients when they had high antibody levels (titer of >1:10,000) against breast cancer extract by ELISA. PBLs were treated to enrich for B-cells and transfected with EBV as described in the methods section. Despite success in the transformation of all of the B-cells, supernatant of only one lymphoblastoid cell line (LCL-4) showed reactivity to breast cancer cell extracts and its spent culture medium in ELISA and Western blot (Figure 7). The band visible in Western blot is of high molecular weight. The immunoglobulin isotype secreted by LCL-4 is IgM.

DISCUSSION:

Our continued efforts in the application of the 90kDa glycoprotein TAA-IC assay have revealed that this marker can indicate the presence of residual disease after surgical resection in breast cancer patients. This is apparent from the observation that 41% post-operative patients were positive for the TAA-IC. Also, longitudenal clinical follow-up of the TAA-IC positive patients revealed that post-operative presence of TAA-IC in serum identified patients that were at high risk of developing recurrence. Clearly, the TAA-IC alone is not a perfect marker because there were some patients (12%) in the post-operative TAA-IC negative group that developed recurrent disease. This could be due to the fact that recurrent tumor of these patients did not express the 90kDa glycoprotein antigen. Furthermore, some patients that were positive post-operatively did not develop recurrent disease for at least up to five years of follow-up. We feel that residual disease in these patients either regressed or remained dormant for the duration of their clinical follow-up. Therefore, it is necessary to identify tumor-associated antigens that are more or less breast cancer specific and are immunogenic in the host.

At the present time breast mammography is recommended for asymptomatic women, every one to two years between the ages of 40 to 49, and once a year for those over 50. However, abnormal mammograms do not necessarily mean the presence of malignant disease. Often, histopathologic examination of biopsy of the lesions revealed them to be benign.

Correlation of the results of histopathologic analysis of biopsy and analysis of the serum taken at the same time for TAA-IC from women with abnormal mammograms, revealed that TAA-IC or similar marker could be a valuable adjunct to mammography. However, as indicated above, like any tumor marker, the TAA-IC marker could not be solely relied upon for clinical judgements in the care of patients until the present results are validated by further studies.

Since the PEG-IC assay is not antigen specific its application to identify sera with high levels of tumor related immune complexes did not prove to be very useful. Instead, we undertook alternative approaches to identify allogeneic antibody rich sera. During the reporting period we have made a significant progress in the direction to identify serum samples of breast cancer patients that exhibit a high level of immunoreactivity to breast cancer cell extracts in ELISA. We selected a serum with an IgG antibody titer of 1:12,800. This serum sample from a breast cancer patient was used to successfully isolate IgG antibodies by the DEAE Affi-Gel blue chromatography. The purity of the antibody was such that no arc other than IgG was seen in immunoelectrophoresis using a goat antiserum directed to whole human serum. Using the purified IgG from sera of breast cancer patient and normal control volunteer, we have identified a band of about 53kDa (B-TAA) in the NP-40 extract of breast cancer cell lines using the Western blot technique. In addition, we have been able to isolate and immortalize B-cells from breast cancer patients. One of the lymphoblastoid cell line (LCL-4) established in this manner continues to produce an IgM antibody that reacts with a band in the high molecular weight region in breast cancer cell extract and its spent culture medium in Western blot and ELISA.

The actual scope of this research project in terms of SOW is smaller than that proposed originally. This was done in response to a communication of July 29, 1994, from the U.S. Army Medical Research Acquisition Activity to accommodate the reviewer's comments of the original proposal. The changed SOW is relisted below:

Task 1. Identify breast cancer associated antigen (B-TAA) that are expressed by breast cancer cells and are immunogenic in breast cancer patients (Months 1-24):

a. Analyze sera from breast cancer patients and control groups in a systematic manner for the levels of IC by PEG-CIC assay to select positive sera.

b. Isolate antibodies (IgG) from sera of breast cancer patients that react specifically with breast carcinoma cells at high titers after absorption with appropriate control cells, and use the antibodies in affinity chromatographic procedures to purify B-TAA from breast carcinoma cells grown in serum free medium.

Task 2. Use the purified B-TAA to develop monoclonal antibodies and B-TAA-specific IC assays (Months 12-36):

a. Develop murine monoclonal antibodies.

b. Develop B-TAA-specific IC detection assay.

c. Isolate B-cells from the blood of breast cancer patients obtained at the time of high antibody levels, transform with Epstein Barr virus, and clone anti-B-TAA antibody producing cells.

Task 3. Use murine and human anti-B-TAA antibodies to develop assays to detect B-TAA in serum of breast cancer patients (Months 36-48):

a. Glycoprotein and B-TAA-specific IC results will be compared to CEA and CA15-3 results.

b. The assay results will be correlated with clinical course (recurrence and treatment) of patients, and used for early detection of breast cancer.

Clearly, up to this point of the project period, we have completed both aspects (1a and 1b) of Task 1, and some aspects of Task 2 and Task 3 of the SOW. Specifically, we have optimized a TAA-specific-IC detection assay that can be successfully applied to identify patients who may be harboring the occult breast cancer (Task 2b). We have isolated and immortalized B-cells from breast cancer patients (Task 2c). At least one of the lymphoblastoid cell line (LCL-4) continues to secrete IgM antibody that reacts with breast cancer cell extracts.

CONCLUSIONS

The 90kDa glycoprotein TAA-specific immune complex detection assay (TAA-IC) which we have optimized in the previous project period has been found to be useful in identifying breast cancer patients who harbor occult micrometastases, or are at high risk of developing recurrent disease. This assay, like most of other tumor markers is not 100% accurate. There are some false postives and some false negatives; however, these false predictive values are less significant than many other markers commonly used. We have studied the immunobiology of the 90kDa glycoprotein TAA to the extent that the false postitive and negative results are reasonably explained. A new B-TAA which is recognized by allogeneic serum samples from breast cancer patients has been identified in NP-40 extract of breast cancer cell lines. A lymphoblastoid cell line (LCL-4) from immortalization of a breast cancer patient's B-cells by EBV transformation has been established. This lymphoblastoid cell line continues to secrete IgM antibodies that react with breast cancer cell extracts and spent medium. In the coming years, our plan is to characterize this B-TAA and utilize it to develop B-TAA-specific-IC detection assay that can be applied in conjunction with the existing TAA-IC for diagnosis and prognosis of breast cancer patients.

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TABLE I: Correlation between 90kDa glycoprotein TAA-IC and post-operative recurrence in breast cancer patients.

Patient* recurrence group (Months)	Number of patients in group	Number of TAA-IC positive patients	Time (months) between TAA-IC positive and recurrence		
			Range	Mean	SD
<12	8	6	1 to 11	6	4
12 - 24	8	6	14 to 20	17	2
25 - 36	13	9	25 to 33	29	3
37 - 48	8	7	37 to 47	40	3
49 - 60	6	6	49 to 58	54	4
All patients	43	34	1 to 58	29	16

* Patients were grouped on the basis of recurrence of disease after removal of primary disease.

TABLE II. Correlation between TAA-specific-IC and histopathologic results of biopsy tissue of patients with abnormal mammography (Total 151 patients).

		90kDa glycoprotein TAA-IC	
Histopathology result	N	Positive	Negative
Ductal Ca in situ	17 (11%)	8 (47%)	9 (53%)
Invasive ductal Ca	59 (39%)	38 (64%)	21 (36%)
No malignancy	75 (50%)	5 (7%)	70 (93%)

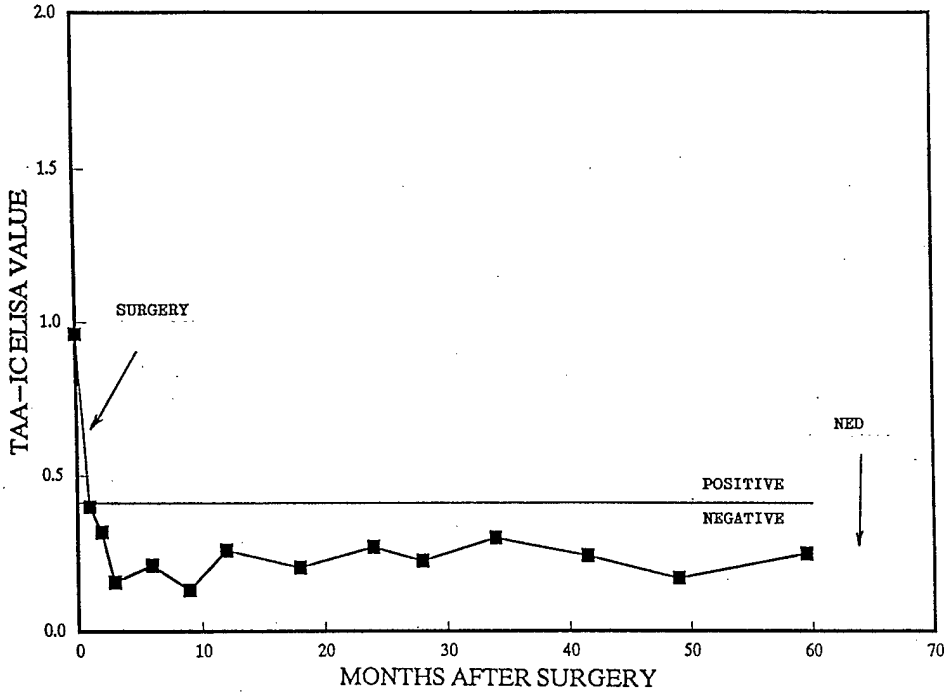

FIGURE 1: 90kDa glycoprotein TAA-specific-IC during the clinical follow-up of a breast cancer patient who remained disease free for more than 5 years. The post-operative serum samples of this patient were in the normal range.

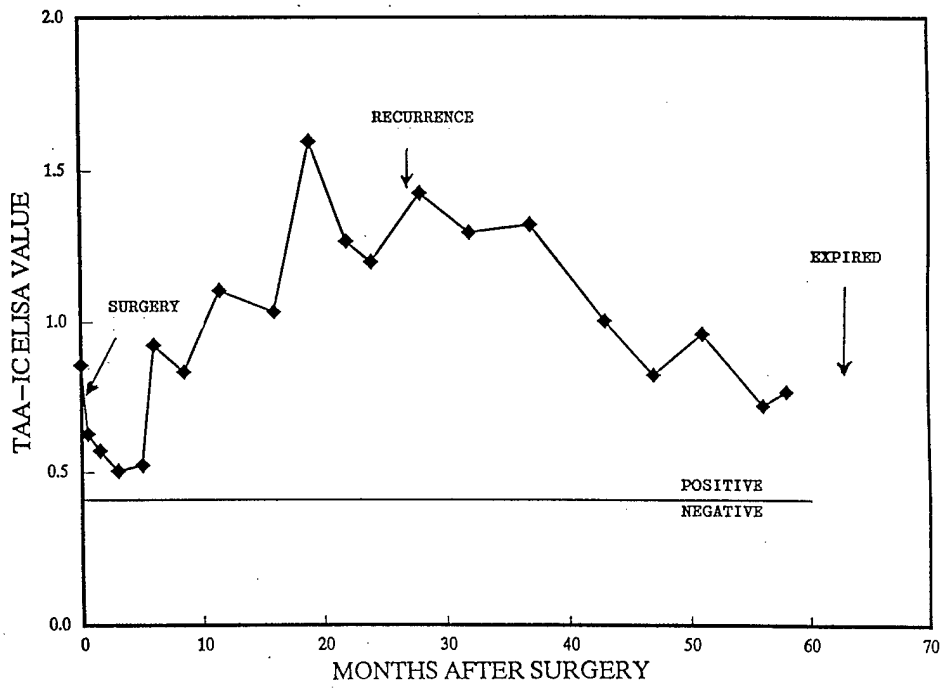

FIGURE 2: 90kDa glycoprotein TAA-specific-IC during the clinical follow-up of a breast cancer patient who developed recurrent disease within 26 months of surgery. The TAA-specific-IC values of sequential serum samples of this patient were positive several months before clinically detectable disease.

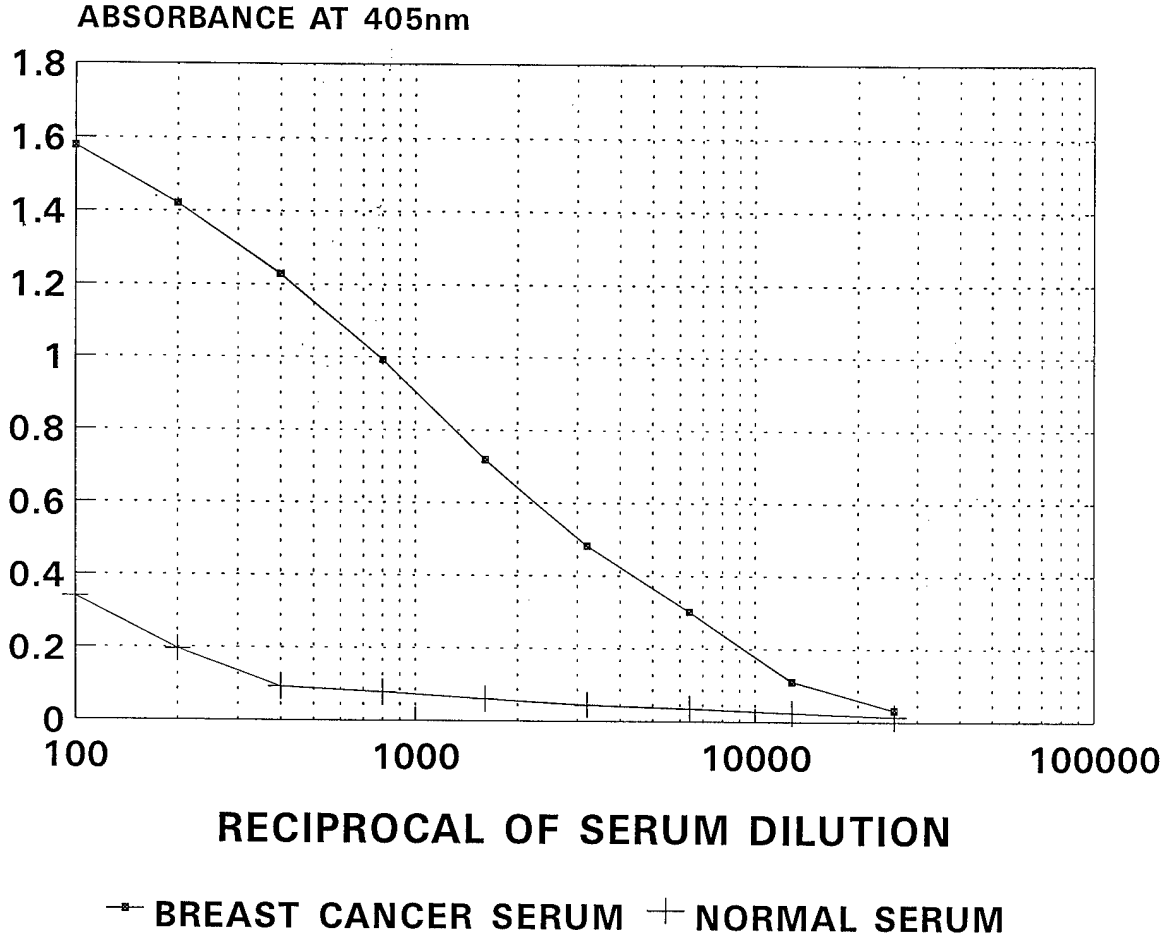

FIGURE 3: Immunoreactivity of representative serum samples from a breast cancer patient and a self-proclaimed normal individual to ultrasonically disrupted tissue culture breast cancer cell extracts in a conventional ELISA. Serum samples were diluted in PBST supplemented with 1% fetal calf serum. Two hundred microgram protein of the cell extract was used as the target per well of the ELISA plate. Alkaline phosphatase conjugated goat anti-human IgG was used as the detecting reagent. A highest dilution of the serum resulting in an optical density of 0.1 at 405_{nm} was considered as the antibody titer.

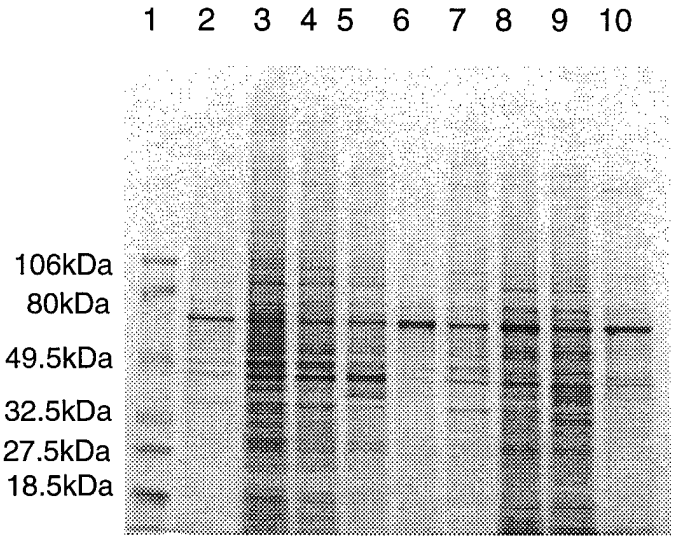

FIGURE 4: SDS-PAGE of NP-40 extracts of various cell line. Extracts were loaded onto lanes of 6 to 20% gradient gel. After electrophoresis, the gels were stained with Coomassie. Lane 1: molecular weight standards; lane 2: melanoma (M14) cell extract; lane 3: breast cancer (157) cell line; lane 4: breast cancer (231) cell line; lane 5: breast cancer (734B) cell line; lane 6: sarcoma (LV) cell line; lane 8: colon (LS174T) cell line; lane 9: breast cancer (Br-2) cell line; and lane 10: normal fibroblast cell line.

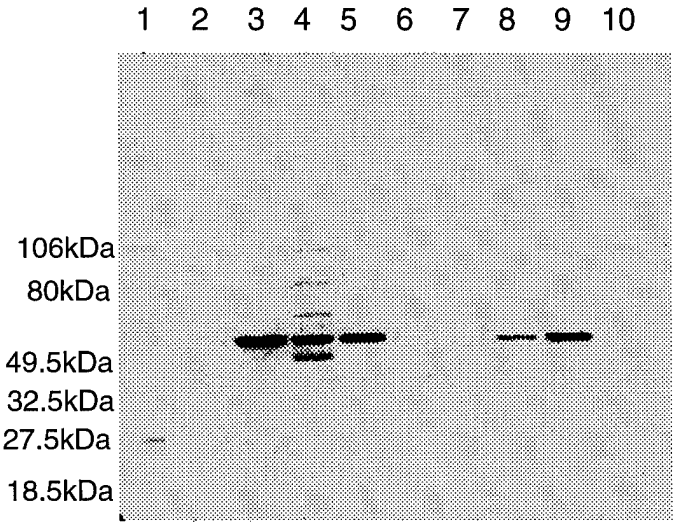

FIGURE 5: Reactivity of purified IgG from serum of a breast cancer patient in Western blot. The IgG was used at a concentration of 3ug/ml, and the reactivity of the antibody was realized using alkaline phosphatase conjugated Fab fragment of goat anti-human IgG. The target in various lanes are as follows: Lane 1: molecular weight standards; lane 2: melanoma (M14) cell extract; lane 3: breast cancer (157) cell line; lane 4: breast cancer (231) cell line; lane 5: breast cancer (734B) cell line; lane 6: sarcoma (LV) cell line; lane 8: colon (LS174T) cell line; lane 9: breast cancer (Br-2) cell line; and lane 10: normal fibroblast cell line.

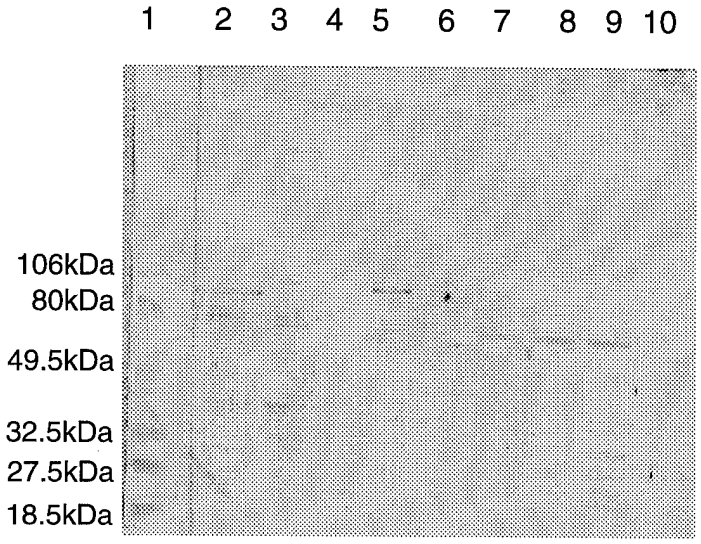

FIGURE 6: Reactivity of normal serum with targets in Figure 4 using Western blot. Similar results were observed when IgG fraction of the normal serum was used as the source of antibody at 200 ug/ml concentration.

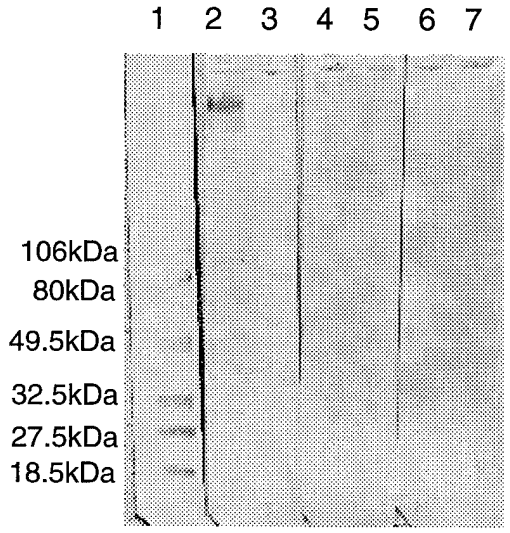

FIGURE 7: Reactivity of LCL-4 culture supernate (antibody source) in Western blot. Lane 1: Molecular weight standards; lane 2: LCL-4 reacted with Br-2 (breast carcinoma cell line) spent medium; lane 3: LCL-4 reacted with normal fibroblast spent medium; lane 4: RPMI instead of LCL-4 culture medium as no antibody control reacted with Br-2 spent medium; lane 5: RPMI instead of LCL-4 culture medium reacted with fibroblast spent medium; lane 6: alkaline phosphatase conjugated goat anti-human IgM control (conjugate control) with spent medium of Br-2; and lane 7: same as lane 6 but against spent medium of normal fibroblasts.